Biochemical Pharmacology, Vol. 37, No. 23, pp. 4445–4449, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

EFFECTS OF PHARMACOLOGICAL MANIPULATIONS ON BASAL AND NEWLY SYNTHESIZED LEVELS OF GABA, GLUTAMATE, ASPARTATE AND GLUTAMINE IN MOUSE BRAIN CORTEX

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(*Received* 16 *January* 1988; *accepted* 3 *May* 1988)

Abstract—Concentrations of basal and newly synthesized inhibitory (γ-aminobutyric acid, GABA) and excitatory (glutamate and aspartate) neurotransmitter amino acids and glutamine were determined in mouse brain cortex. Isotopic enrichment following an intravenous infusion of a stable-labeled precursor, [$^{13}C_6$]D-glucose, was used to estimate the newly synthesized amino acid content. Effects of various pharmacological agents (valproate, aminooxyacetic acid, 3-mercaptopropionic acid, *N*-methyl-D-aspartate, and 2-amino-7-phosphonohepatanoic acid) were evaluated. The effects of 3-mercaptopropionic acid (an inhibitor of glutamate decarboxylase, a GABA-synthesizing enzyme) were restricted to the GABAergic system. On the other hand, *N*-methyl-D-aspartate (an agonist of a glutamate receptor subtype) was selective for the glutamate-glutamine system, and its effects were prevented by its selective antagonist, 2-amino-7-phosphonoheptanoic acid. In some cases, divergent effects were observed on basal and new amino acids. This suggested that basal and new amino acids may represent different compartments. The anticonvulsant drug valproate caused an increase in basal but a decrease in newly synthesized GABA. Aminooxyacetic acid caused a dramatic increase in basal GABA without affecting the newly synthesized GABA. This approach may be useful in studying compartmentation and fluxes of neurotransmitters.

There is growing evidence supporting the importance of inhibitory and excitatory amino acid neurotransmitters in experimental animal models of epilepsy [1]. The balance between inhibitory and excitatory neurotransmission may be important in the pathogenesis and potential treatment of epilepsy [2]. GABA (γ-aminobutyric acid) is the principal inhibitory amino acid [3], whereas aspartate (ASP) and glutamate (GLU) [4] are the excitatory amino acids that have been implicated in the epileptogenesis or as targets in the treatment of epilepsy [5].

Due to compartmentation, total tissue concentrations of neurotransmitters (including amino acids) may not be indicators of active neurochemical processes but may be more representative of the net overall static situation [6–10]. Functional neuronal activity and the related dynamic changes may be better represented by turnovers or concentrations of the newly synthesized neurotransmitters [8–11].

Administration of a labeled precursor, which leads to an isotopic enrichment of a neurotransmitter, can be utilized to measure newly synthesized amino acids and their turnovers [12]. Infusion of uniformly labeled [^{13}C]glucose results in incorporation of a $^{13}C_2$-labeled fragment into neurotransmitter amino acids in the brain, probably through acetate via the tricarboxylic acids [12].

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This study examined the effects of pharmacological perturbations on the total tissue content of amino acids and their isotopic enrichment following the infusion of stable-labeled glucose. The purpose of the study was 2-fold: (a) to determine if pharmacological manipulations have differential effects on naturally occurring and isotopically-enriched amino acids and (b) to evaluate if the observed changes were consistent with existing neuropharmacological knowledge. The study concentrated on the three major neuroactive amino acids, GABA, ASP, and GLU, along with glutamine (GLN) which is closely related metabolically. Terms "basal" and "new" are used here to denote concentrations of naturally occurring and isotopically enriched amino acids respectively.

EXPERIMENTAL

Chemicals. Natural isotopes of L-amino acids were obtained from Sigma (St. Louis, MO). The deuterated amino acids, used as internal standards for the analysis, and [$^{13}C_6$]D-glucose were purchased from MSD Isotopes (St. Louis, MO). All labeled compounds used had greater than 98% isotopic enrichment. Pentafluoropropionic anhydride and ethyl alcohol were purchased from Pierce (Rockford, IL) and 3.0 M hydrochloric acid in *n*-butanol was obtained from Regis (Morton Grove, IL). Deionized water was obtained using the Milli-Q reagent-grade water system (Millipore, Bedford, MA). Organic

solvents were purchased from Burdick & Jackson Laboratories (Muskegon, MI). Aminooxyacetic acid (AOAA), 3-mercaptopropionic acid (3MPA) and *N*-methyl-D-aspartate (NMDA) were obtained from Sigma, 2-amino-7-phosphonoheptanoic acid (APH) was from Research Biochemicals Inc. (Wayland, MA) and valproic acid (VPA) was from Saber Laboratories (Morton Grove, IL). Other chemicals were of the best commercially available grades.

Animals. Male NIH general purpose mice (25–30 g) were individually weighed in order to adjust the doses of pharmacological agents. All drugs were administered in saline (0.01 ml/g body weight), but it was necessary to use sodium hydroxide to adjust the pH of VPA to 7.5 in order to facilitate its dissolution. Details about the doses, routes and times of administration (prior to glucose infusion) are described in the figure legends. Subsequent to administration of pharmacological agent(s) or saline (control), all animals received [$^{13}C_6$]D-glucose (44.8 μmol in 0.2 ml saline) intravenously via the tail vein by constant infusion over 9.25 min (syringe infusion pump 22, Harvard Apparatus, South Natick, MA). Immediately at the end of the infusion, the mice were decapitated and the heads were cooled rapidly by swirling in physiological saline at −0.5° for 2 min to minimize postmortem changes [13]. The brain was then excised and placed on a chilled aluminium plate kept on ice. A brain section was obtained from each hemisphere with two transverse razor blade cuts, the first immediately caudal to the olfactory bulb and the second 3 mm caudal to the first. Cortical tissue (about 25 mg), mainly representing the frontal cortex, was quickly peeled away from the underlying structures. The tissue was weighed and immediately frozen in a polypropylene tube using a dry ice–acetone bath and stored overnight at −20°.

Analysis. The details of the analytical method have been published elsewhere [14]. Briefly, tissue samples were homogenized in cold 80% ethyl alcohol (100 μl/mg wet tissue weight) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After centrifugation, the supernatant fraction was stored in the refrigerator. Deuterated internal standards ([$^2H_6$]GABA, [$^2H_5$]GLU and [$^2H_5$]GLN) were added to 100 μl of the supernatant fraction, and the samples were applied on 1-ml Bond-Elut (Analytichem International, Harbor City, CA) cartridges containing 100 mg of strong anion-exchange packing (SAX; trimethylaminopropyl) for isolation of the dicarboxylic amino acids followed by strong cation-exchange packing (SCX; benzenesulfonylpropyl) for isolation of the neutral amino acids. After elution and evaporation, the extracts were reacted first with 3 N hydrochloric acid in *n*-butanol and then with pentafluropropionyl anhydride to yield *n*-butyl ester pentafluropropionyl amide derivatives. The derivatives were analyzed by gas chromatography–mass spectrometry (Hewlett–Packard model 5987A, Palo Alto, CA) using a methane positive chemical ionization mode after gas chromatographic separation on a fused-silica (5% phenylmethyl DB5, J&W Scientific, Folsom, CA) capillary column. Quantitation was based on the peak heights of the quasi-molecular ions using selected ion monitoring. Isotopic enrichment was determined after the subtraction of the contribution from naturally occurring isotopes. Concentrations of the newly synthesized amino acids were calculated based on the concentrations of basal amino acids and the percent isotopic enrichment.

RESULTS

The data in Fig. 1 depict effects of the anticonvulsant drug (VPA) on the concentrations of basal and new GABA, ASP, GLU, and GLN at two different time points. VPA caused a statistically significant increase in basal and a decrease in new GABA levels. In addition, VPA decreased basal and new ASP levels, the effect being more pronounced on the new ASP. These effects were more dramatic at 2 min than at 30 min following VPA pretreatment. This time course was consistent with our observation on VPA (i.p.) protection against s.c. pentylenetetrazole-induced seizures (unpublished results), i.e. significantly greater protection at 2 versus 30 min. Both basal GLU and basal GLN concentrations were also elevated at the earlier time point.

The effects of AOAA in Fig. 2 show a dramatic elevation in basal GABA levels without a significant effect on the newly synthesized GABA. AOAA also decreased newly synthesized ASP and basal GLN.

The effects of 3MPA were evaluated in VPA-pretreated animals and compared to a VPA-saline control group (Fig. 3). VPA was used in order to prevent 3MPA seizures and thereby avoid secondary seizure-induced effects. The only statistically significant effect of 3MPA was on the GABAergic system.

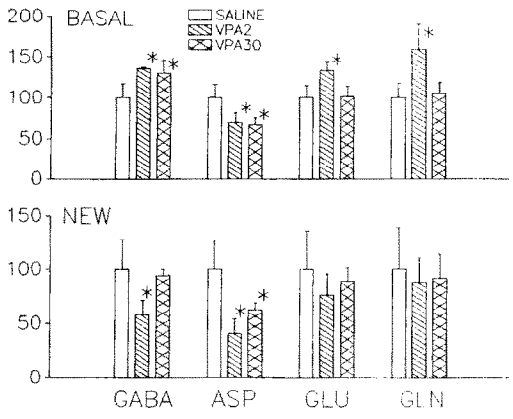

Fig. 1. Effect of VPA on brain cortical concentrations of basal and new amino acids. VPA (200 mg/kg) was administered as an i.p. bolus injection 2 min (VPA2) or 30 min (VPA30) prior to labeled glucose infusion. Mean and SD data are presented as a percent relative to the concurrent control group for 15, 3 and 7 animals in control (SALINE), VPA2, and VPA30 groups respectively. Control group values for basal amino acids in μmol/g wet tissue weight (mean ± SD) were: GABA, 1.50 ± 0.26; ASP, 5.41 ± 0.89; GLU, 10.66 ± 1.64; and GLN, 4.73 ± 0.86. Control group values for new amino acids in μmol/g wet tissue weight (mean ± SD) were: GABA, 0.086 ± 0.024; ASP, 0.156 ± 0.041; GLU, 0.741 ± 0.257; and GLN, 0.110 ± 0.043. Key: (*) different from saline control group, $P < 0.05$ (two-tailed Dunnett's *t*-test).

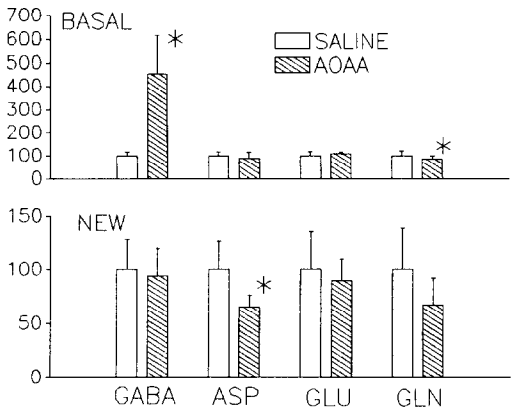

Fig. 2. Effect of AOAA on brain cortical concentrations of basal and new amino acids. AOAA (30 mg/kg) was administered as an i.p. bolus injection 60 min prior to labeled glucose infusion. Control animals received saline. Mean and SD data are presented as a percent relative to the concurrent control group for 15 animals in the SALINE and 7 animals in the AOAA group. Control group values for basal amino acids in μmol/g wet tissue weight (mean ± SD) were: GABA, 1.50 ± 0.26; ASP, 5.41 ± 0.89; GLU, 10.66 ± 1.64; and GLN, 4.73 ± 0.86. Control group values for new amino acids in μmol/g wet tissue weight (mean ± SD) were: GABA, 0.086 ± 0.024; ASP, 0.156 ± 0.041; GLU, 0.741 ± 0.257; and GLN, 0.110 ± 0.043. Key: (*) different from saline control, $P < 0.05$ (two-tailed Student's t-test).

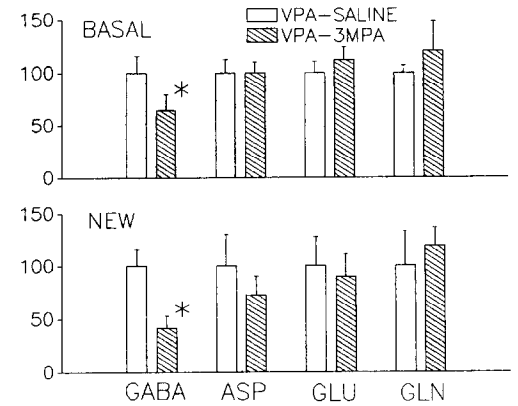

Fig. 3. Effect of 3MPA on brain cortical concentrations of basal and new amino acids in VPA-pretreated mice. All animals were pretreated with valproic acid (350 mg/kg i.p. bolus injection 20 min prior to labeled glucose) to prevent seizures by 3MPA (35 mg/kg i.p. bolus injection) which was administered 10 min prior to labeled glucose infusion. Control animals (VPA-SALINE) received VPA followed by a saline injection. Mean and SD data are presented as a percent relative to the concurrent control group for 4 animals in VPA-SALINE and 3 in VPA-3MPA group. Control group values for basal amino acids in μmol/g wet tissue weight (mean ± SD) were: GABA, 2.08 ± 0.33; ASP, 4.30 ± 0.54; GLU, 11.05 ± 1.17; and GLN, 5.28 ± 0.38. Control group values for new amino acids in μmol/g wet tissue weight (mean ± SD) were: GABA, 0.058 ± 0.009; ASP, 0.065 ± 0.019; GLU, 0.510 ± 0.136; and GLN, 0.055 ± 0.018. Key: (*) different from saline control group, $P < 0.05$ (two-tailed Student's t-test).

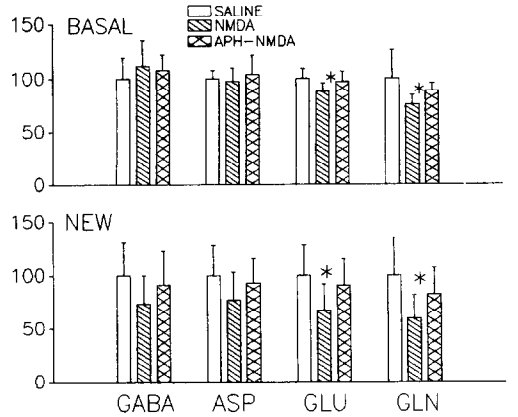

Fig. 4. Effect of NMDA on brain cortical concentrations of basal and new amino acids. NMDA (72 mg/kg) was administered as a subcutaneous bolus 5 min prior to labeled glucose infusion. Control animals received saline. One group of animals (APH-NMDA) also received APH (50 mg/kg) as an i.p. bolus 30 min prior to NMDA (35 min prior to labeled glucose). Mean and SD data are presented as a percent relative to the concurrent control group for 8, 7, and 8 animals in SALINE, NMDA and APH-NMDA groups respectively. Control group values for basal amino acids in μmol/g wet tissue weight (mean ± SD) were: GABA, 2.01 ± 0.39; ASP, 5.00 ± 0.39; GLU, 10.77 ± 0.98; and GLN, 5.67 ± 1.46. Control group values for new amino acids in μmol/g wet tissue weight (mean ± SD) were: GABA, 0.110 ± 0.033; ASP, 0.124 ± 0.034; GLU, 0.624 ± 0.171; and GLN, 0.116 ± 0.040. Key: (*) different from saline control group, $P < 0.05$ (two-tailed Dunnett's t-test).

Both basal and new GABA were reduced, the effect being more pronounced on the newly synthesized GABA.

The effects of NMDA and NMDA following APH were selective for the GLU-GLN system and are summarized in Fig. 4. Statistically significant reduction in both basal and new GLU and GLN was observed following NMDA with the effect more pronounced on the newly synthesized amino acids. The effects of NMDA were prevented by APH pretreatment; the levels of basal and new GLU and GLN were not statistically different from those of the saline control group.

DISCUSSION

VPA is an anticonvulsant drug, and its exact mechanism of action has not been, as yet, ascertained. In search of its mechanism of action, various neurochemical effects of VPA were studied. VPA has been reported to decrease the activity of GABA-T (GABA transaminase, a GABA-degrading enzyme) [15], increase activity of GAD (glutamic acid decarboxylase, a GABA-synthesizing enzyme) [15], and stimulate glutamine synthetase [16]. Perlman and Goldstein [17] reported that VPA is a very potent membrane disordering agent and suggested that some of its effects may be a result of this action. It was shown here that a pharmacological dose (200 mg/kg) of VPA increased basal GABA, GLU and GLN, whereas it decreased basal ASP. The

effect of VPA on new GABA was completely the opposite to that observed for basal GABA, a reduction in new GABA with no effect on new GLU. Differential effects of VPA on basal and new GABA suggest that these two parameters may represent two different pools of GABA. This effect of VPA on new GABA could be explained if VPA, directly or indirectly, facilitated GABAergic transmission and thereby decreased the synthesis of the neuroactive GABA via feedback inhibition. Similar findings were reported by Chapman *et al.* [18] in rats following 400 mg/kg sodium valproate, perhaps a neurotoxic dose [19]. These authors showed that VPA reduces the rate of labeling of GABA without affecting the rate of GLU labeling following an i.v. pulse dose of [2-^{14}C]glucose. They reported effects of VPA on basal amino acids similar to ours. Loscher and Vetter [19] showed that VPA increases GABA levels in both whole tissue and synaptosomes from rat brain cortex.

AOAA has dose-dependent anticonvulsant properties. It is a relatively non-specific GABA-T inhibitor, but since it interacts with the coenzyme pyridoxal phosphate, it affects a number of enzymes [20]. Loscher and Vetter [19] reported that AOAA (30 mg/kg) increases GABA levels in rat cortical synaptosomes and whole tissue. In this study, we observed a large increase in the basal GABA without any effect on the new GABA. Differential effects on the concentrations of basal and new GABA are again suggestive that these two parameters reflect different GABA pools. After an intracerebroventricular injection of [^{14}C]GLU and [^{3}H]GABA, Abe and Matsuda [11] have shown that AOAA increases total tissue GABA and newly taken up GABA without an effect on newly synthesized GABA. On the basis of their data, they proposed the existence of two GABA pools in nerve terminals. One pool is associated with GAD and consists of newly synthesized GABA in cytoplasm near the presynaptic membrane. This newly synthesized GABA is preferentially released on stimulation. The other pool is associated with GABA-T and mainly represents newly taken up GABA in synaptic vesicles, possibly near mitochondria. On the basis of the data of Abe and Matsuda as well as his own, Loscher [21] proposed that compounds which inhibit GABA-T would show minimal effectiveness in improving GABA function in disorders caused by GABAergic deficiency.

3MPA is a GAD inhibitor and causes seizures by decreasing synaptic GABA [22–25]. It may also increase GABA-T activity [22, 23]. 3MPA was shown to decrease whole tissue and synaptosomal GABA levels in rat brain cortex [21]. We have found that the 3MPA effects were selective for the GABAergic system decreasing both basal and new GABA, the effect being more pronounced for the newly synthesized GABA.

NMDA is an agonist of an excitatory dicarboxylic amino acid receptor subtype [26]. The excitatory dicarboxylic amino acids, GLU and ASP, have been proposed to play an important role in learning and memory processes, neurodegenerative disorders, cerebral ischemia and epilepsy [27, 28]. In this study, we examined the effects of a subconvulsive dose of NMDA and found statistically significant effects selective for the GLU-GLN system. NMDA caused a decrease in basal and new GLU and GLN, a greater effect being seen on the newly synthesized amino acids. Prior pretreatment with APH, a selective NMDA antagonist which blocks NMDA seizures, prevented the occurrence of NMDA-related effects.

The presence of compartmentation in brain synaptosomes has been proposed for acetylcholine [29] and GABA [11]. Therefore, even though synaptosomal concentrations of neurotransmitter amino acids may offer more information than the whole tissue levels, specific activity of the functional neurotransmitter pool may still be obscured. This could explain the similar effects of VPA and AOAA on synaptosomal and whole tissue levels of GABA [19]. As proposed earlier [11], the newly synthesized neurotransmitter may be a more sensitive index of neuronal activity than synaptosomal content since, in some cases, nerve endings appear to contain more than one neurotransmitter pool with different functional significance.

This preliminary study examined the effects of pharmacological manipulations on three amino acid neurotransmitters implicated in the cause or prevention of seizures. Qualitatively and quantitatively distinguishable effects of pharmacological manipulations on basal and new amino acids may be indicative of different compartments. However, additional studies are needed to further evaluate the usefulness of newly synthesized amino acids as a parameter for examining pools and fluxes of the neurotransmitters and the relation to neuronal activity.

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